Surface plasmon resonance analysis was carried out as described Moestrup, S. K. *et al.* β_2-glycoprotein-I (apolipoprotein H) and β_2-glycoprotein-I- phospholipid complex harbor a recognition site for the endocytic receptor megalin. *J. Clin. Invest* **102**, 902-909 (1998). Purified CD163 was immobilised at the BIAcore sensor CM5 chip (BIAcore AB) at a concentration of up to 50 µg/ml in 10 mM sodium acetate, pH 4.0, and the remaining binding sites were blocked with 1 M ethanolamine pH 8.5. The surface plasmon resonance signal generated from immobilised CD163 corresponded to 55 – 66 fmol receptor/mm^2. The sample and flow buffer was 10 mM Hepes, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 7.4. The sensor chips were regenerated with 1.6 M glycine-HCl, pH 3. The binding assay for measuring binding of ^{125}I-Hp-Hb to human CD163 immobilised in microtiter plate wells (Nunc) was carried out as described Birn, H. *et al.* Characterization of an epithelial approximately 460-kDa protein that facilitates endocytosis of intrinsic factor-vitamin B12 and binds receptor-associated protein. *J. Biol. Chem.* **272**, 26497-26504 (1997).

The microtiter plates were coated at 4°C for 20 h with purified CD163 in 50 mM $NaHCO_3$ containing 250 ng CD163 per well (for binding ^{125}I-Hp(1-1)-Hb) or 125 ng CD163 per well (for binding ^{125}I-Hp(2-2)-Hb). Iodination of Hp-Hb was performed with the chloramine-T-method. Ligand blotting was carried out as described using 10^6 cpm radioligand/ml (Moestrup, S. K. & Gliemann, J. Analysis of ligand recognition by the purified α_2-macroglobulin receptor (low density lipoprotein receptor-related protein). Evidence that high affinity of α_2-macroglobulin-proteinase complex is achieved by binding to adjacent receptors. *J. Biol. Chem.* **266**, 14011-14017 (1991).α

Hp is synthesised as a single chain, which is post-translationally cleaved into an amino-terminal α chain and a carboxy-terminal β chain. The basic structure of Hp, as found in most mammals, is a homodimer (Fig. 2a), in which the two Hp molecules are linked by a single disulfide bond via their respective ~9 kDa α chains[14]. In man, a variant with a long α chain is also present in all populations. This variant arose apparently by an early intragenic duplication, presumably originating from an unequal crossover of two basic alleles, resulting in an Hp with an α chain of ~14 kDa. The short and long α chains are designated as α1 and α^2, respectively. Since the cysteine forming the intermolecular disulfide bond between the α chains is also duplicated, humans carrying the long variant allele exhibit a multimeric Hp phenotype (Fig. 2a).

Analysis of the binding of Hp-Hb complexes (Fig. 2a) to immobilised CD163 revealed a high-affinity binding of both dimeric and multimeric Hp-Hb complexes (Fig. 2b and c). Fig. 2b shows a surface plasmon resonance analysis of CD163 binding of the dimeric Hp(1-1)-Hb complex and the multimeric Hp(2-2)-Hb complex. No binding of non-complexed Hb (Fig. 2b, left panel) nor Hp(1-1) or Hp(2-2) (Fig 2b, middle and right panels) was detected thus indi-

cating that a neoepitope for receptor binding is expressed in the Hp-Hb complex. Accordingly, maximal receptor binding was measured, when the Hb binding capacity of Hp reached saturation (Fig. 2b, middle and right panels) at equimolar concentrations of Hb and Hp. The Hp(2-2)-Hb complex yielded a higher response and the dissociation was slower as compared to the Hp(1-1)-Hb complex. The results shown were obtained using the A_0 ($\alpha_2\beta_2$) form of Hb. Similar results were obtained using the A_2 ($\alpha_{2\delta 2}$) form or the S form (Hb with the mutation for sickle cell disease) [15] (data not shown).

Example 3

Binding affinity

A solid phase assay with immobilised CD163 in microtiter wells was used for various inhibition experiments (Fig. 6c). This analysis revealed that the removal of Ca^{2+} with EDTA or the addition of polyclonal anti-CD163 IgG completely abolished the binding of Hp-Hb to CD163. Measuring the true affinity of the one-site interaction of Hp-Hb binding to CD163 was hampered by the suggested divalency (Hp(1-1)) and multivalency (Hp(2-2)) of the ligand in terms of receptor-recognition sites. However, competition for CD163-binding of ^{125}I-labelled Hp-Hb by unlabelled Hp(1-1)-Hb and Hp(2-2)-Hb complexes showed, as anticipated from the surface plasmon resonance experiments, an ~10 fold higher functional affinity (avidity) of the multimeric Hp(2-2)-Hb complexes (Fig. 6c). The concentration of unlabelled Hp(1-1)-Hb complex causing 50% inhibition of the binding of ^{125}I-labelled Hp(1-1)-Hb was ~0.3 µg/ml, giving an 'apparent K_d' of ~2 nM of the dimeric Hp(1-1)-Hb complex. In contrast, the 50% inhibition point for Hp(2-2)-Hb was at ~0.1 µg/ml giving an 'apparent K_d' of ~0.2 nM (on assumption of the 2-2 multimer distribution previously calculated Wejman, J. C., Hovsepian, D., Wall, J. S., Hainfeld, J. F. & Greer, J. Structure and assembly of haptoglobin polymers by electron microscopy. *J. Mol. Biol.* **174**, 343-368 (1984).). The higher functional affinity of the 2-2 type complex is probably accounted for by its higher valency. Similar 'bonus effect of multivalency' is well known in other biological systems, e.g. the binding of the pentameric IgM molecule to several identical surface antigens.

Example 4

Endocytosis analysis in CD163-transfected CHO cells and in SU-DHL cells

The cDNA encoding the most abundant variant of CD163 (Genbank/EMBL accession no Z22968) Law, S. K. *et al.* A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. *Eur. J. Immunol.* **23**, 2320-2325 (1993) was ligated into

the KpnI and NotI sites of the mammalian expression vector pcDNA3.1/Zeo(+) (Invitrogen). Stable transfected CHO clones expressing CD163 were established by limited dilution and selection with 500 µg/ml Zeocin (Invitrogen). Expression products were analysed by immunoblotting of growth medium and cell lysate using the rabbit polyclonal antibody against the ligand-affinity purified human CD163.

Endocytosis of ^{125}I-Hp-Hb in CD163-tranfected and mock-transfected CHO cells growing as confluent adherent monolayers in 24-well plates was analysed as previously described Moestrup, S. K. & Gliemann, J. Analysis of ligand recognition by the purified α_2-macroglobulin receptor (low density lipoprotein receptor-related protein). Evidence that high affinity of α_2-macroglobulin-proteinase complex is achieved by binding to adjacent receptors. *J. Biol. Chem.* **266**, 14011-14017 (1991). Endocytosis in the soluble SU-DHL-1 histiocytic lymphoma cells (2 x 10^6 cell/ml) was analysed as described Moestrup, S. K., Christensen, E. I., Sottrup-Jensen, L. & Gliemann, J. Binding and receptor-mediated endocytosis of pregnancy zone protein-proteinase complex in rat macrophages. *Biochim. Biophys. Acta* **930**, 297-303 (1987).

CD163-mediated endocytosis of ^{125}I-Hp-Hb complexes was studied in Chinese Hamster Ovary (CHO) cells transfected with CD163 cDNA (the abundant CD163 form, Genbank/EMBL accession no Z22968). Fig. 7a (middle panel) shows the time course of cell-associated radioactivity and trichloroacetic acid (TCA)-soluble radioactivity (representing degraded ligand) in the medium. The cell-associated radioactivity reached a plateau after one hour of incubation, and about this time, the TCA-soluble radioactivity significantly increased in the medium. Consistent with an endocytic uptake of Hp-Hb, a similar experiment conducted in the presence of the lysosomal inhibitors, chloroquine and leupeptin, showed a continual increase in cell-bound radioactivity for 3 hours with essentially no TCA-soluble radioactivity detected (Fig. 7a, right panel).

The endocytosis of Hp-Hb complexes was mediated by CD163, since no uptake, and consequently no TCA-soluble radioactivity, was detected in incubations with CHO cells not expressing the CD163 antigen (Fig. 7a, left panel). Furthermore, uptake and degradation of ^{125}I-labelled Hp(2-2)-Hb can be inhibited by purified IgG from anti-CD163 serum and by unlabelled Hp(2-2)-Hb complexes (Fig. 7b, left panel). Similar results (Figure 7b, right panel) were obtained with the myelo-monocytic SU-DHL-1 cell line (Epstein, A. L. *et al.* Biology of the human malignant lymphomas. IV. Functional characterization of ten diffuse histiocytic lymphoma cell lines. *Cancer* **42**, 2379-2391 (1978), the only cell line Pulford, K., Micklem, K., Law, S. K. & Mason, D. Y. in Leukocyte Typing VI. (eds. Kishimoto, T. *et al.*) 1089-1091 (Garland Publishing Inc, New York, 1997) known to express the CD163 antigen, and with